
In the Name of God

Inhibitory effect of MyoD on the proliferation of breast cancer cells

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ONCOLOGY LETTERS 11: 3589-3596, 2016

Inhibitory effect of MyoD on the proliferation of breast cancer cells

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Received January 11, 2015; Accepted February 24, 2016

DOI: 10.3892/ol.2016.4448

Abstract. Skeletal muscle is rich in lymphatic vessels, with an abundant blood supply, and it is an infrequent site of cancer metastasis. Previous studies have demonstrated that enhanced secretion of MyoD may occur when skeletal muscle is injured or becomes cancerous. It was hypothesized that MyoD may act as an endogenous cytokine to inhibit the proliferation of cancer cells. To verify the possible effect of this protein on tumor cell proliferation, C2C12 mouse skeletal muscle cells and 4T1 mouse breast cancer cells were co-cultured using embedded Transwell plates. Following co-culture, cell cycle analysis revealed that C2C12 muscle cells were able to inhibit the proliferation of the breast cancer cells. Subsequently, MyoD was silenced in C2C12 cells to assess its effect on 4T1 cell proliferation. Following co-culture with MyoD-silenced cells, a 5-ethynyl-20-deoxyuridine assay indicated that MyoD silencing prevented the reduction in proliferation of 4T1 cells induced by untransfected C2C12 cells. In summary, the results indicated that MyoD inhibits the proliferation of breast cancer cells and may be a tumor suppressor factor.

Introduction

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Introduction

MyoD was first cloned in 1987 and termed MyoD1 (1). The protein has a basic helical three-dimensional crystal structure containing a basic helix-loop-helix domain that is able to bind to other proteins that also possess this domain, including myocyte-enhancing factor 2, myogenin and creatine kinase (CK). Its adjacent basic region is required for it to bind to the promoters or enhancers of numerous muscle-specific genes, including CK and myogenin (1,2). The N-terminus of MyoD contains a histidine-cysteine domain and a transcription activation domain, which are associated with the transcriptional activation of MyoD target genes, and the C-terminus contains a facultative helical (helix III) domain that may be associated with chromatin remodeling (3-5).

As a member of the muscle transcription factor family, MyoD has decisive roles in muscle differentiation, including muscle conversion and the maintenance of muscle differentiation (1,2). Recent studies have demonstrated that a synthetic MyoD polypeptide has a high affinity for the inhibitor of DNA-binding proteins (ID), and thus may inhibit the binding of ID with DNA, thereby inhibiting the proliferation of cancer cells (6). In addition, Dey *et al* (7) identified that MyoD is an important cytokine in cerebellar development and a tumor suppressor gene in medulloblastoma. These previous studies strongly indicate the existence of a close association between MyoD and cancer cells.

As a major organ, skeletal muscle is rich in lymphatic vessels with an abundant blood supply. However, few studies have demonstrated cancer metastasis to skeletal muscle tissue (8-12). MyoD expression may be increased following skeletal muscle injury or its invasion by cancer cells (13,14). The present study aimed to test the hypothesis that MyoD may act as an endogenous cytokine to inhibit the growth of metastatic cancer. Its expression was assessed in breast cancer tissue and cell lines and in C2C12 skeletal muscle cells, and the proliferation of breast cancer cells was evaluated following co-culture with control or MyoD-silenced skeletal muscle cells.

Materials and methods

Cell culture and co-culture. The immortalized mouse myoblast cell line C2C12 and the mouse breast tumor cell line 4T1 (each gifted by the Xiangya Central Experiment Laboratory, Changsha, China) were maintained at 37˚C in an atmosphere of 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 100 U/ml penicillin, 100 U/ml

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Key words: skeletal muscle, metastatic cancer, MyoD, breast cancer

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Materials and methods

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Transwell chambers (0.4-μm pore size; Corning Incorporated, Corning, NY, USA) were placed into 6-well plates. The interior of the Transwell plate was designated the upper chamber, while the space between the plates formed the lower chamber, and the chambers were separated by a polycarbonate membrane. Due to the permeability of the polycarbonate membranes, components in the lower-layer medium are able to affect the growth and movement of cells

placed in the upper chamber. In order to study the impact of cytokines secreted by skeletal muscle cells on cancer cells, Transwell chambers were used to form a co-culture, with skeletal muscle cells in the lower chamber and cancer cells in the upper chamber (15). C1C12 and 4T1 cells were firstly cultured in a culture flask to at a cell concentration of $5x10^5$/ml for ~48 h until they reached 70% confluence. The C2C12 cells were subsequently transplanted onto a 6-well plate (Corning Incorporated) for 24 h, and the 4T1 cells were cultured in Transwell (Corning Incorporated). The cells were co-cultured

Table I. siRNA sequences and properties.

siRNA	Sequences	GC%
01	5'-GCCUGAGCAAAGUGAAUGA-dTdT-3'	39
	3'-dTdT-CGGACUCGUUUCACUUACU-5'	39
02	5'-CAGCAGACGACUUCUAUGA-dTdT-3'	39
	3'-dTdT-GUCGUCUGCUGAAGAUACU-5'	39
03	5'-CCAACUGCUCUGAUGGCAU-dTdT-3'	43
	3'-dTdT-GGUUGACGAGACUACCGUA-5'	43

Materials and methods

- *Cell culture and co-culture*
- *Immunohistochemical analysis*
- *Immunofluorescence*
- *Small interfering RNA (siRNA) synthesis and transfection*
- *Semi-quantitative RT-PCR*
- *Western blotting*
- *Cell cycle analysis using propidium iodide (PI) and flow cytometry*
- *5-ethynyl-20-deoxyuridine (EdU) assay*
- *Statistical analysis*

different groups were washed three times with ice-cold PBS and then lysed in lysis buffer [50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1% Triton X-100 and 100 μg/ml phenylmethylsulfonyl fluoride] on ice for 20 min. Following centrifugation at 16,000 x g for 2 min at 4˚C, supernatants were separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and then electrophoretically transferred to nitrocellulose membranes (Invitrogen; Thermo Fisher Scientific, Inc.). After blocking for 2 h with 5% fat-free milk at room temperature, the membranes were incubated with the primary mouse monoclonal anti-MyoD antibody or control mouse monoclonal anti-β-actin secondary antibody (dilution, 1:1,000; #A1978; Sigma-Aldrich China, Inc.) for 24 h at 4˚C. The membranes were then incubated with a secondary biotinylated goat anti rabbit IgG polyclonal antibody (dilution, 1:1,000; #A6667; Sigma-Aldrich China, Inc.) for 1 h at room temperature. Protein bands were visualized using Pierce enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.) and Odyssey v1.2 software (LI-COR Biosciences, Lincoln, NE, USA). The intensity of expression was measured by comparing the target and control bands.

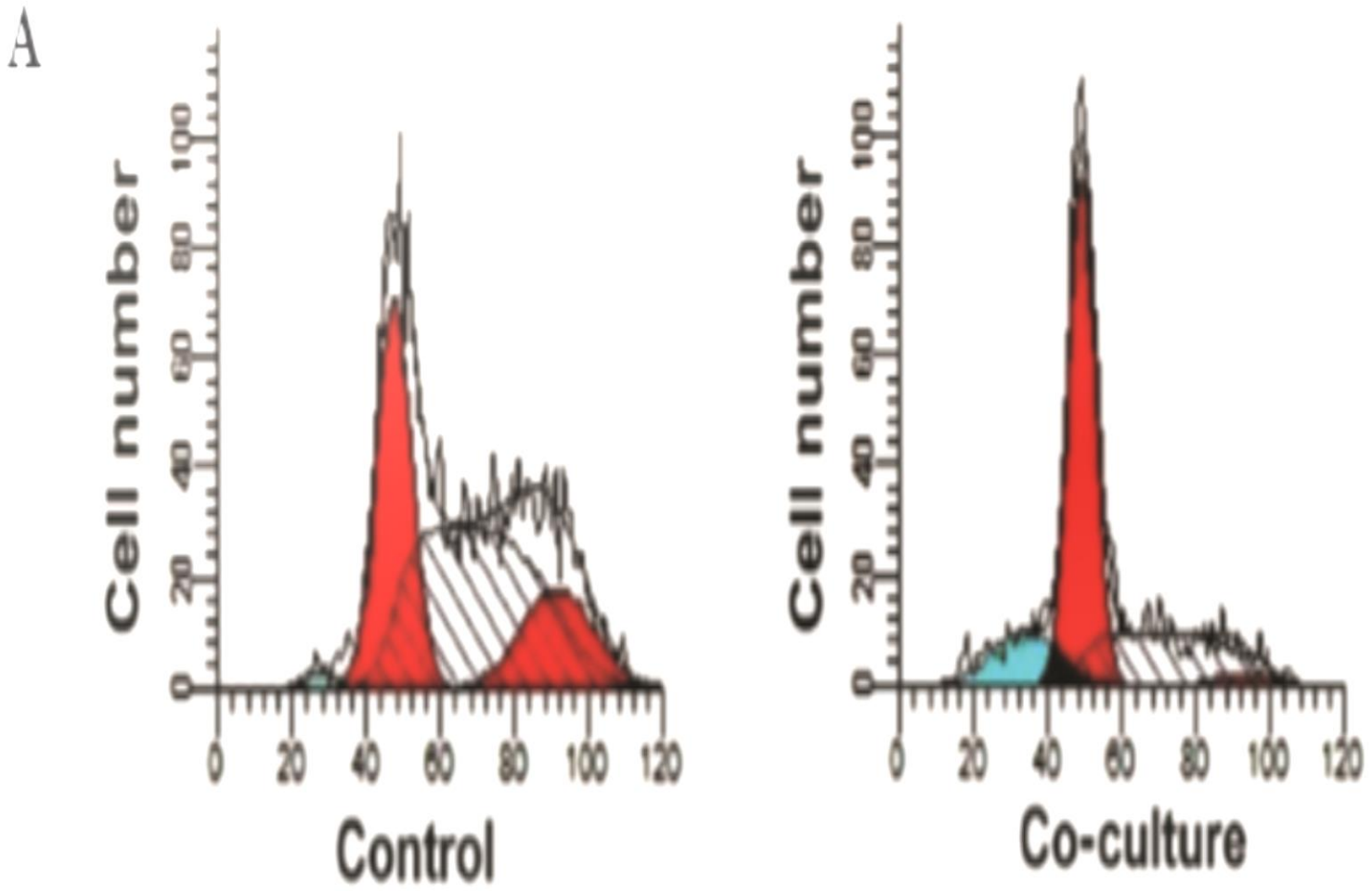
Cell cycle analysis using propidium iodide (PI) and flow cytometry. Cell cycle analysis was conducted at 72 h after transfection. 4T1 cells ($5x10^5$) from the tested groups were harvested by brief trypsinization, washed twice with PBS, fixed in 70% ethanol overnight and stained with PI (final concentration, 20 mg/ml)/Triton X-100 solution containing 10 mg/ml

were considered significant when $P<0.05$. The unpaired *t*-test was used for comparing two groups. All statistical analyses were performed using SPSS software version 18 (SPSS, Inc., Chicago, IL, USA).

Results

Proliferation of 4T1 cells may be suppressed by C2C12 cells. To identify whether skeletal muscle cells can inhibit the proliferation of cancer cells, mouse breast tumor cells (4T1 cells) and mouse myoblast cells (C2C12 cells) were co-cultured on Transwell plates. Mouse breast tumor cells and mouse breast tumors were co-cultured as controls. PI staining and flow cytometry were used to detect the proliferation of the 4T1 cells. As shown in Fig. 1, at 48 h after co-culture, 65% of the cells were in G1 phase and 33% were in S phase in the experimental group, compared to 31% in G1 phase and 56% that were in S phase in the control group (G1: P=0.0376 vs. control group; S: P=0.0396 vs. control group; G2/M: P=0.0479 vs. control group; n=6), demonstrating that the proliferation of the 4T1 cells was inhibited following co-culture with the C2C12 cells (Fig. 1A and B).

Silencing efficiency of MyoD siRNAs. RNA interference was used to generate a C2C12 cell line with targeted silencing of MyoD. To verify the silencing efficiency of MyoD siRNA, three candidate siRNAs were designed and numbered 01, 02 and 03, respectively. The expression of MyoD mRNA in C2C12 cells was detected using RT-PCR following siRNA transfec-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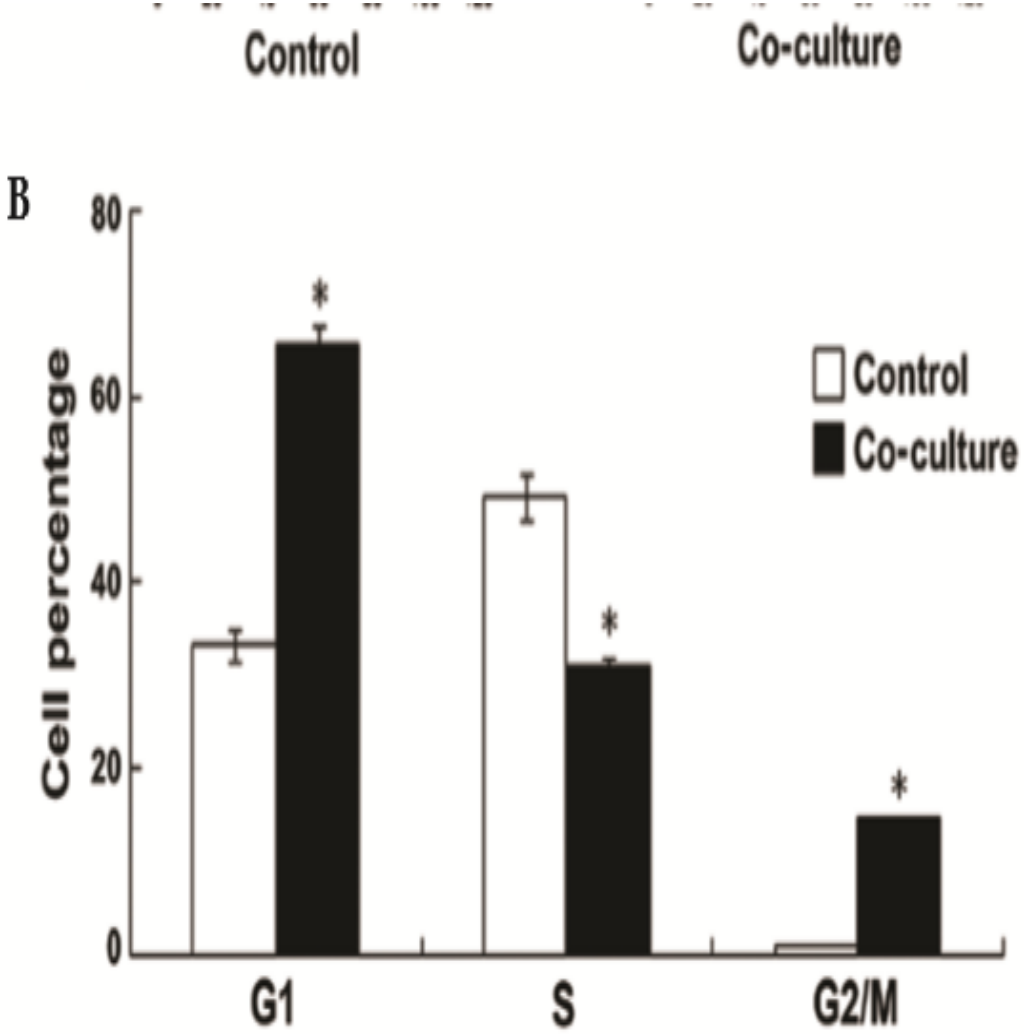
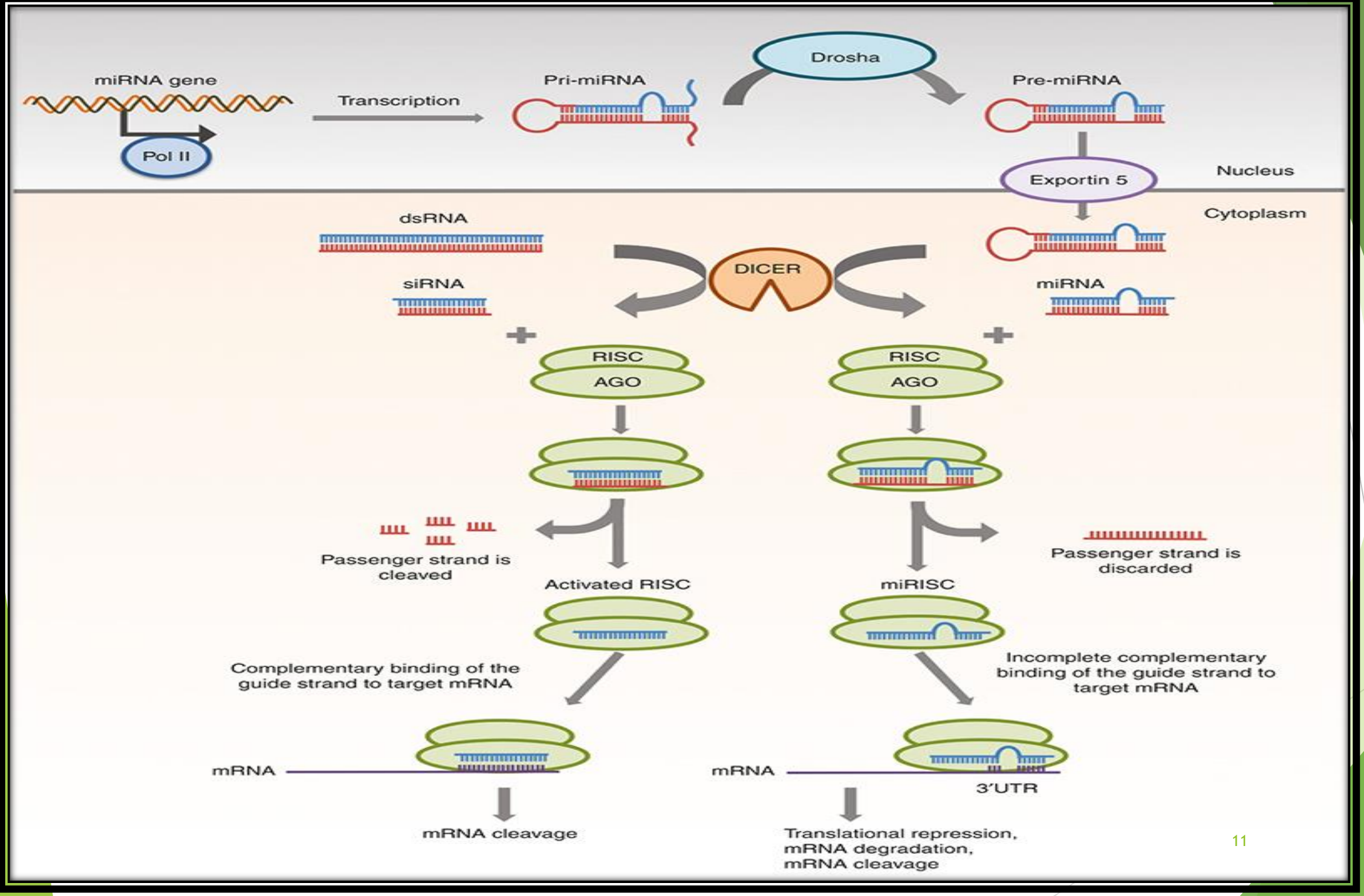


Figure 1. Proliferation of 4T1 cells was suppressed by co-culture with C2C12 skeletal muscle cells. (A) After 48 h of co-culture, the populations of cells in G1, S and G2/M phases from the different groups were separated and counted. (B) Cell populations in the G1, S and G2/M phases are shown in histograms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Transcription
Pri-miRNA
Drosha
Pre-miRNA
Exportin 5
Nucleus
Cytoplasm
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siRNA
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RISC
AGO
RISC
AGO
Passenger strand is cleaved
Activated RISC
miRISC
Passenger strand is discarded
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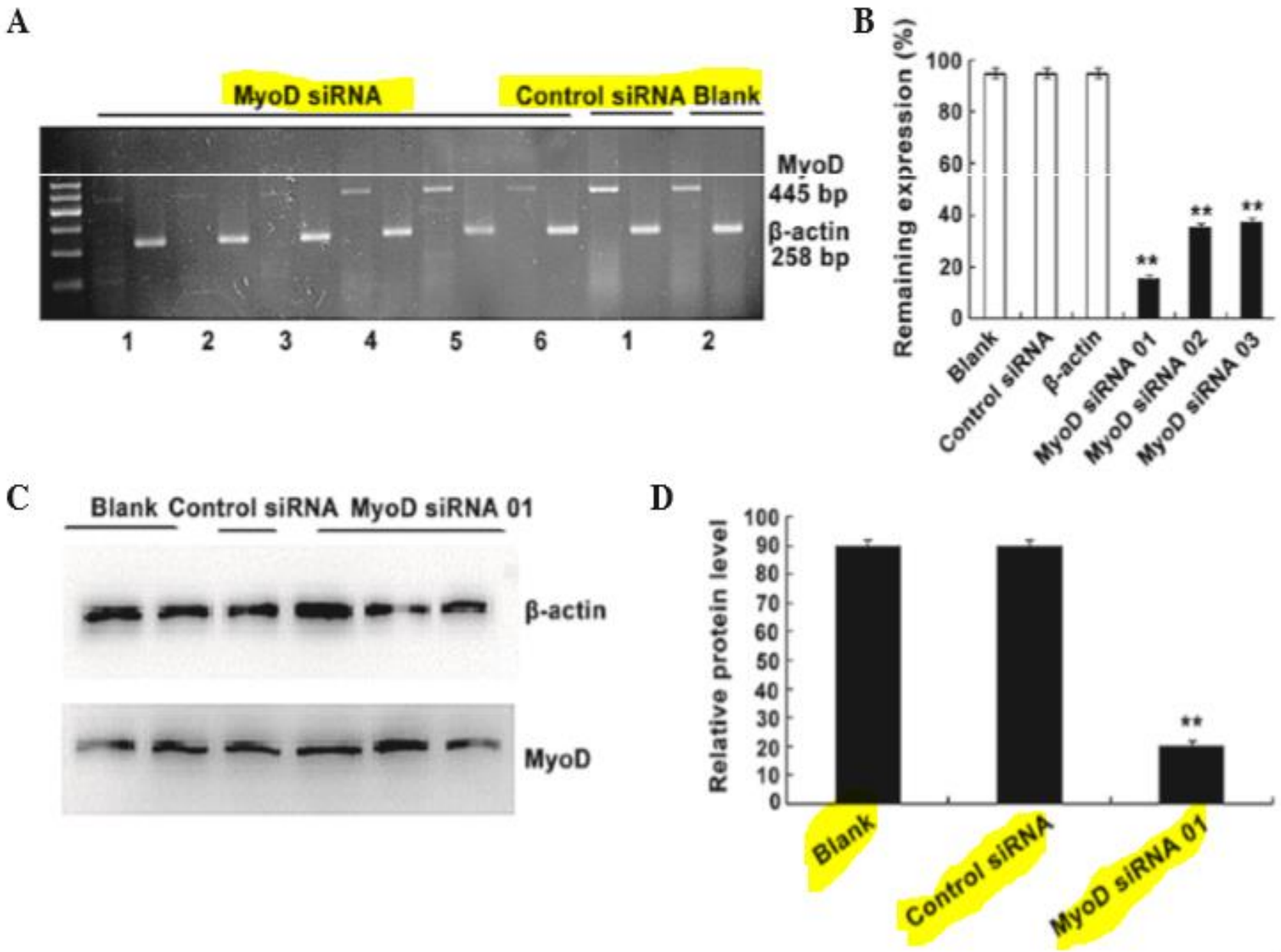


ure 2. Silencing efficiency of MyoD siRNAs. (A) Silencing of MyoD mRNA in C2C12 cells. Three candidate MyoD siRNAs [siRNA 01 (lanes 1-3
NA 02 (lanes 4-5) and siRNA 03 (lane 6)], and 100 nmol of non-silencing control siRNA was transfected into C2C12 cells in triplicate. Semi-quantitativ
PCR was conducted at 48 h after siRNA transfection. (B) Semi-quantification of RT-PCR normalized to β-actin levels (**P<0.01 vs. control). (C) The mo

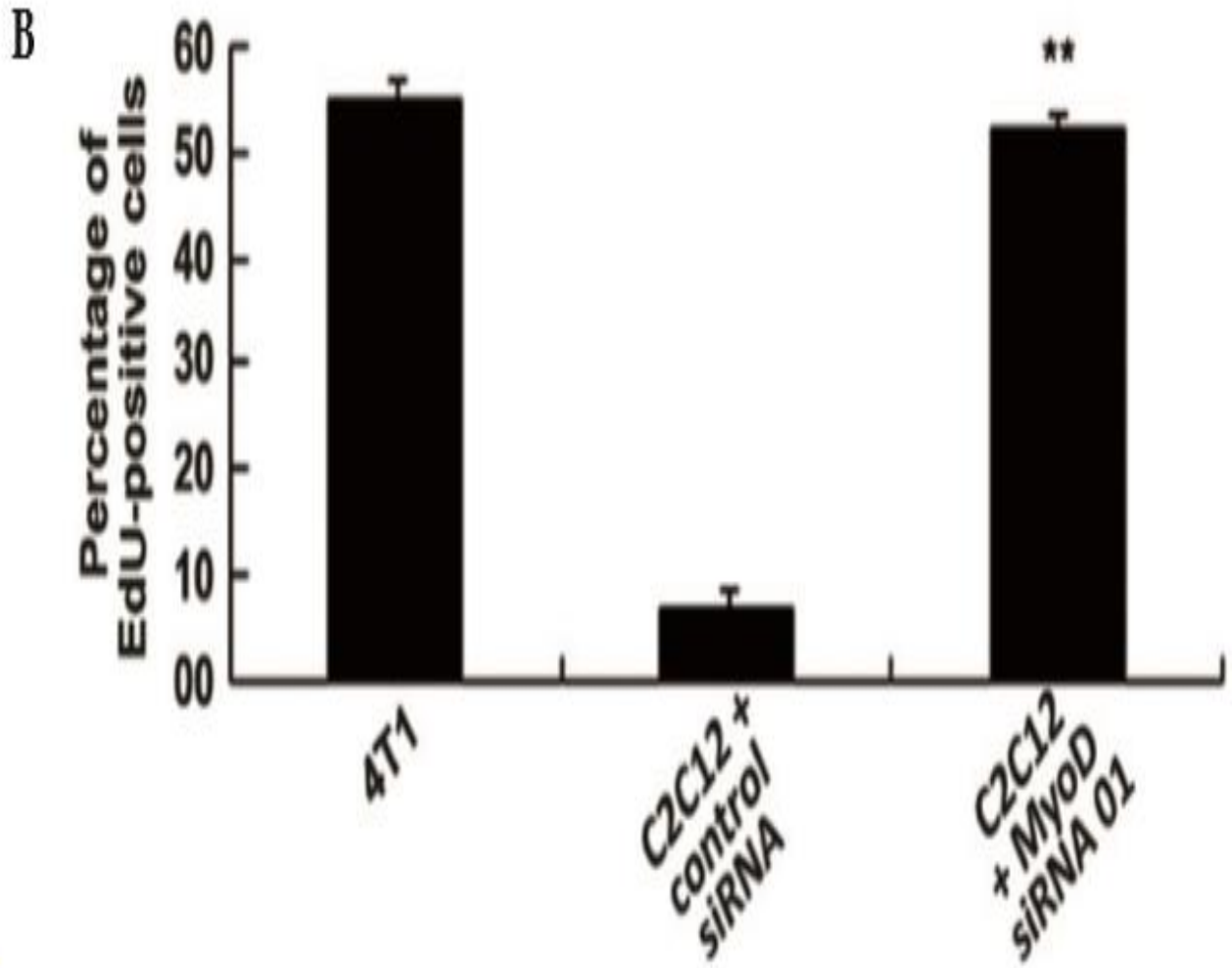
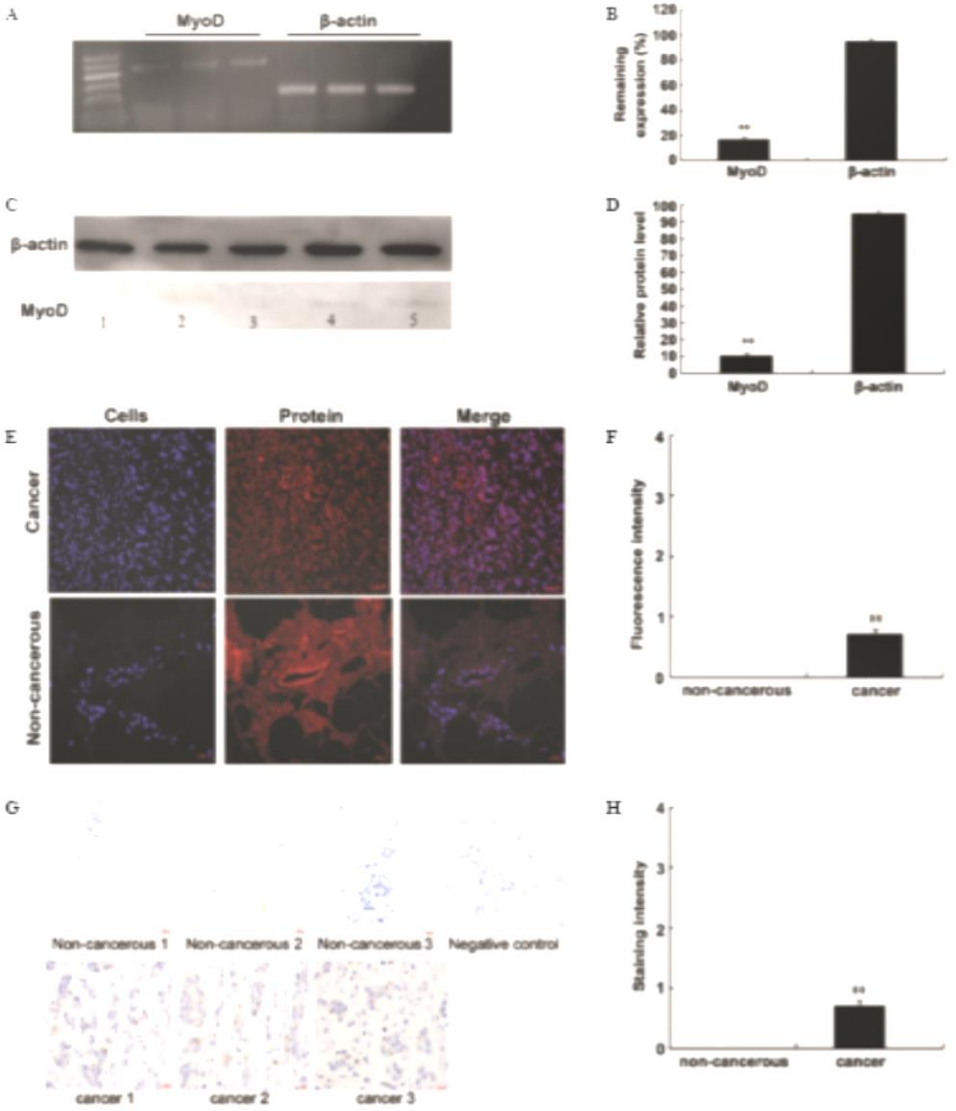


Figure 3. Proliferation of 4T1 cells can be inhibited by MyoD. (A) 4T1 cells were labeled with EdU following co-culture with MyoD siRNA-transfected or control siRNA-transfected C2C12 cells, or with only 4T1 cells, in Transwell chambers. The Click-iT reaction revealed EdU staining (red), and cell nuclei were stained with Hoechst 33342 (blue). The images are representative of the results obtained. (B) The percentage of EdU-positive 4T1 cells was quantified. The data are presented as the mean ± standard error of the mean. **P<0.01 vs. 4T1.



the expression of MyoD in the tissues. The other difference between the control tissues and the cancer may be due to the atypia of cancer cells. To the best of our knowledge, this is the first report of low levels of MyoD expression in breast cancer tissue.

Discussion

Skeletal muscle is widely distributed and is an infrequent site of cancer metastasis (9). The current study was conducted to test the hypothesis that an endogenous tumor suppressor factor

may be associated with the low incidence of cancer metastasis in skeletal muscle. MyoD secretion increases when skeletal muscle is injured or invaded by cancer cells, suggesting that there is an association between MyoD and tissue wound repair (1). We hypothesized that MyoD may be an endogenous tumor suppressor factor that is also associated with the low occurrence of cancer in skeletal muscle (16).

MyoD is a DNA-binding protein that also has a significant role in skeletal muscle differentiation due to its importance in muscle conversion (6). Recently, Dey *et al* (7) demonstrated

indicated that MyoD may act as a tumor suppressor gene in 4T1 cells; however, this conclusion requires further verification. In addition, the low incidence of skeletal muscle metastasis is not limited to one type of cancer cell, suggesting that MyoD may be a suppressor of multiple types of cancer (8,9).

MyoD also serves important roles in muscle transformation in the skeletal muscle microenvironment. Several previous studies have reported the successful transformation of fat cells into skeletal muscle cells by MyoD transfection *in vitro* (22),

may be associated with the low incidence of cancer metastasis in skeletal muscle. MyoD secretion increases when skeletal muscle is injured or invaded by cancer cells, suggesting that there is an association between MyoD and tissue wound repair (1). We hypothesized that MyoD may be an endogenous tumor suppressor factor that is also associated with the low occurrence of cancer in skeletal muscle (16).

MyoD is a DNA-binding protein that also has a significant role in skeletal muscle differentiation due to its importance in muscle conversion (6). Recently, Dey *et al* (7) demonstrated that MyoD is an important cytokine during cerebellar development and is a tumor suppressor gene in medulloblastoma. In fact, MyoD may regulate gene expression as a DNA-binding protein. Chen *et al* (6) used synthetic peptide fragments of MyoD to block the binding of DNA with ID, which is an important regulator of cell proliferation. After ID binding is blocked, cancer cell proliferation decreases (17), indicating a possible pathway by which MyoD may inhibit the proliferation of these cells. However, MyoD is a large protein, and its ability to enter the cell and affect DNA duplication require further verification (18,19).

As it is difficult to monitor the biological activity of MyoD *in vitro*, a MyoD-silenced model of mouse myoblast C2C12 cells was constructed in the present study. The C2C12 cells were co-cultured with 4T1 mouse breast cancer cells in

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In conclusion, the current study demonstrates for the first time that MyoD plays a critical role in cancer development by inhibiting the proliferation of cancer cells. Furthermore, it may act as a tumor suppressor gene in multiple types of cancer cells. These results will aid in the elucidation of the mechanisms underlying the low incidence of cancer metastasis in skeletal muscle.

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Acknowledgements

The authors would like to thank the Department of Pathology of Xiangya Hospital for their support in obtaining the specimens. In addition, this work was supported by NSFC (grant nos. 81170016, 81270065, 81370116 and 81570026), Hunan Natural


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FOR YOUR ATTENTION